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T-cell receptor gene transfer exclusively to human CD8+ cells enhances tumor cell killing

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Abstract: Transfer of tumor-specific T-cell receptor (TCR) genes into patient T cells is a promising strategy in cancer immunotherapy. We describe here a novel vector (CD8-LV) derived from lentivirus, which delivers genes exclusively and specifically to CD8(+) cells. CD8-LV mediated stable in vitro and in vivo reporter gene transfer as well as efficient transfer of genes encoding TCRs recognizing the melanoma antigen tyrosinase. Strikingly, T cells genetically modified with CD8-LV killed melanoma cells reproducibly more efficiently than CD8(+) cells transduced with a conventional lentiviral vector. Neither TCR expression levels, nor the rate of activation-induced death of transduced cells differed between both vector types. Instead, CD8-LV transduced cells showed increased granzyme B and perforin levels as well as an up-regulation of CD8 surface expression in a small subpopulation of cells. Thus, a possible mechanism for CD8-LV enhanced tumor cell killing may be based on activation of the effector functions of CD8(+) T cells by the vector particle displaying OKT8-derived CD8-scFv and an increase of the surface density of CD8, which functions as coreceptor for tumor-cell recognition. CD8-LV represents a powerful novel vector for TCR gene therapy and other applications in immunotherapy and basic research requiring CD8(+) cell-specific gene delivery.

DOI: https://doi.org/10.1182/blood-2012-02-412973

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ZORA URL: https://doi.org/10.5167/uzh-68467
Journal Article
Published Version

Originally published at:
Zhou, Qi; Schneider, Irene C; Edes, Inan; Honegger, Annemarie; Bach, Patricia; Schönfeld, Kurt; Schambach, Axel; Wels, Winfried S; Kneissl, Sabrina; Uckert, Wolfgang; Buchholz, Christian J (2012). T-cell receptor gene transfer exclusively to human CD8+ cells enhances tumor cell killing. Blood, 120(22):4334-4342.
DOI: https://doi.org/10.1182/blood-2012-02-412973

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blood

2012 120: 4334-4342
Prepublished online August 16, 2012;
doi:10.1182/blood-2012-02-412973

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.
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T-cell receptor gene transfer exclusively to human CD8$^+$ cells enhances tumor cell killing

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Transfer of tumor-specific T-cell receptor (TCR) genes into patient T cells is a promising strategy in cancer immunotherapy. We describe here a novel vector (CD8-LV) derived from lentivirus, which delivers genes exclusively and specifically to CD8$^+$ cells. CD8-LV mediated stable in vitro and in vivo reporter gene transfer as well as efficient transfer of genes encoding TCRs recognizing the melanoma antigen tyrosinase. Strikingly, T cells genetically modified with CD8-LV killed melanoma cells reproducibly more efficiently than CD8$^+$ cells transduced with a conventional lentiviral vector. Neither TCR expression levels, nor the rate of activation-induced death of transduced cells differed between both vector types. Instead, CD8-LV transduced cells showed increased granzyme B and perforin levels as well as an up-regulation of CD8 surface expression in a small subpopulation of cells. Thus, a possible mechanism for CD8-LV enhanced tumor cell killing may be based on activation of the effector functions of CD8$^+$ T cells by the vector particle displaying OKT8-derived CD8-scFv and an increase of the surface density of CD8, which functions as coreceptor for tumor-cell recognition. CD8-LV represents a powerful novel vector for TCR gene therapy and other applications in immunotherapy and basic research requiring CD8$^+$ cell-specific gene delivery. (*Blood*. 2012;120(22):4334-4342)

Introduction

In the human body, a vast diversity of immune cells constantly patrols the blood stream and tissues to protect from invaders. Each type of these immune cells fulfills different functions. Genetic modification of these cells is a key technology to elucidate their physiologic functions and to develop novel therapeutic strategies. Among the different types of gene vector systems available, γ-retroviral and lentiviral vectors (LVs) have become state-of-the-art technology for lymphocyte gene transfer.[1-3] Failure to distinguish between subtypes of cells and thereby transferring genes to both target and nontarget cells is a limitation of vector systems currently in use. Selective and specific delivery of transgenes into particular types of lymphocytes is highly desirable for immunotherapy and gene therapy. Although few attempts have been undertaken to retarget LVs to CD3$^+$ T cells,[4] the transduction of subpopulations or even the transfer of therapeutic genes by such targeting vectors has not been described. In addition, no targeting vector specific for CD8$^+$ T cells has been described. CD8$^+$ T cells are among the most important immune cell types and also a primary target for immunotherapy because of their capacity to directly engage and kill pathogen infected cells or tumor cells.[5] Adoptive transfer of tumor-specific T cells is a promising strategy of directed tumor cell killing, which is currently under investigation in clinical trials worldwide.[6-9] Tumor specificity is provided by an antigen receptor, which can be natural (T-cell receptor; TCR) or engineered (chimeric antigen receptor, CAR). Whereas TCR gene-modified T cells recognize peptide-major histocompatibility complex (pMHC), CAR recognize antigen in an MHC-independent fashion. In each case, the receptor encoding sequence is transferred into patient T cells, which are then amplified to generate a large number of tumor-specific T cells for cancer treatment. With current technology, TCR genes are transferred into all types of T cells as the vectors do not distinguish between T-cell subtypes, such as CD4$^+$ and CD8$^+$ cells.

We recently developed a flexible and highly specific method to restrict LV-mediated gene transfer to a cell type of choice and demonstrated proof-of-principle by generating vectors specific for hematopoietic stem cells, B lymphocytes, endothelial cells, and neurons.[10] The technology relies on engineered glycoproteins of measles virus (MV), which are the hemagglutinin (H) and fusion (F) proteins, both incorporated into the envelope membrane of LV particles.[10] Cell-type specificity is provided through a single-chain antibody (scFv) that recognizes a cell-surface antigen selectively expressed on the cell type of interest fused to an engineered H protein, which is blinded for its natural receptors CD46 (complement regulatory protein) and CD150 (signaling lymphocyte activation molecule, SLAM). The extension of this technology to other target cell types of interest relies on the availability of suitable scFv. These must not only be specific for the target cell but also have to be efficiently expressed on the surface of the packaging cells as H protein fusion to become readily incorporated into vector particles.[11]

In this study, we generated a CD8-specific scFv that allows LV-mediated gene delivery using the CD8 surface molecule as receptor for cell entry. This vector, CD8-LV, transferred genes

Submitted February 23, 2012; accepted July 12, 2012. Prepublished online as *Blood* First Edition paper, August 16, 2012; DOI 10.1182/blood-2012-02-412973.

There is an Inside *Blood* commentary on this article in this issue.

The online version of this article contains a data supplement.

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exclusively into the $CD8^+$ subset of T cells, in vitro as well as in vivo. Expression of melanoma-reactive TCRs exclusively in $CD8^+$ T cells significantly enhanced tumor-cell killing compared with conventional gene transfer mediated by a nontargeted LV.

Methods

Plasmid constructions

The CD8-scFv coding sequences were amplified by reverse transcription of OKT8 hybridoma mRNA followed by PCR using the heavy and light primer mix (GE Healthcare) as previously described.[10] The CD8-scFv CDR coding regions were grafted into the HuCAL consensus frameworks huV_H1 and $huV_L\kappa1$,[12] the resulting sequence was synthesized with optimized codons (Geneart), and inserted into pCG-Hmut backbone via *Sfi*I and *Not*I.[10]

Transfer vector plasmids encoding the TCRs T58 and D115, both specific for the melanocyte antigen tyrosinase$_{369\text{-}377}$ (YMDGTMSQV), were constructed by excision of the transgene cassette from the γ-retroviral vector MP71 and subcloning the coding sequences into the lentiviral vector pRRL.PPT.MPpre. This vector is derived from pRRL.PPT.MP.GFPpre, which is a third generation LV[13] with an internal MPSV (myeloproliferative sarcoma virus) U3 promoter, which is particularly active in lymphocytes.[14,15] To construct pRRL.PPT.MP.GFPpre, the MPSV U3 was amplified via PCR using primers 5′ MPSV nhe (5′-GAGCTAGCTTAAGTAAGCCATTTTGCAAGG-3′; restriction sites underlined) and 3′ MPSV bam age (5′-GACCGGTGGATCCCGGGCCCGCGGTACCCCGGGCGAC-3′) on retroviral vector template pMP71.[16] The resulting *Nhe*I/*Age*I fragment was introduced into pRRL.PPT.SF.GFPpre[17] to substitute for the internal promoter of this vector. Sequence details are available on request. Because the constant regions of both TCR chains were replaced by murine counterparts, the transgenic TCR can be distinguished from endogenous TCR by anti–mouse constant region of TCR β-chain (mCβ) monoclonal antibody (mAb) staining.[18]

Cell lines and primary cells

Cultivation of HEK293T cells has been described.[19] The T-cell lines Molt 4.8, Jurkat 76 deficient of endogenous CD8 expression (J76), J76 stably transduced with CD8α (J76CD8),[20] and J76CD8 stably transduced with SLAM (J76CD8SLAM), were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine (L-Glu). J76CD8SLAM cells were generated by transduction of J76CD8 cells with the retroviral vector MP71 encoding SLAM (CD150). Bulk culture was stained with antibodies and revealed more than 85% of CD8 and SLAM double-positive cells. The human melanoma cell lines, Mel-624.38 (HLA-$A2^+$, Tyr^+),[21] Mel-A375 (HLA-$A2^+$, Tyr^-, ATCC: CRL-1619), and SK-Mel-28 (HLA-$A2^-$, Tyr^+, ATCC: HTB-72), were cultured as described.[9] Human PBMCs were isolated, cultivated, and activated as described.[22] Primary $CD8^+$ T cells were isolated by depletion of $CD8^-$ cells using a $CD8^+$ T cell isolation kit (Miltenyi Biotec). Purified cells were stimulated for 3 days in 24-well plates precoated with anti-CD3 (1 μg/mL) and anti-CD28 (1 μg/mL) mAbs (BD Bioscience). Cells were then cultured in RPMI 1640 medium supplemented with 10% FBS, 2mM L-Glu, 1mM HEPES, 100 U/mL penicillin-streptomycin, and 100 IU/mL IL-2 (Roche).

Vector particle production and titration

Vector particles were generated as described[10] using polyethylenimine (PEI) for transfection of HEK293T cells. To produce CD8-LV, 1.5×10^7 cells were cotransfected with 1.9 μg of pCG-H-α$CD8^{opt}$, 9.3 μg of F protein expression plasmids pCG-FcΔ30,[19] 28.7 μg of HIV-1 packaging plasmid pCMVΔR8.9, and 30.2 μg of the transfer vector plasmid pSEW encoding GFP, or the TCR-encoding transfer vector plasmids pLV-T58 and pLV-D115, respectively. MV-LV and VSV-G-LV were produced as described.[19] After 2 days, vector particles released into the cell supernatant were concentrated and purified by ultracentrifugation through a 20% (wt/vol) sucrose cushion (120 000*g* for 3 hours at 4°C). The supernatant was discarded and pellets were resuspended in 100 μL OptiMEM or PBS.

To determine the titers of CD8-LV and VSV-G-LV stocks, Molt 4.8 cells were transduced by at least 4 serial dilutions of vector particles. GFP^+ cells or transgenic TCR^+ cells stained by anti-mCβ mAb were quantified by flow cytometry. Titer calculation was based on those dilutions showing a linear correlation with the dilution factor. Typical titers for CD8-LV ranged between 5×10^6 and 2×10^7 transduction units (tu)/mL.

Transduction of cells and antibody competition assay

For transduction, 1×10^5 cells were incubated with vector particles and 4 μg/mL protamine sulfate (Sigma-Aldrich) in a final volume of 300 μL for 3 hours before 1 mL fresh medium was added. After 2 days, transgene expression was determined by flow cytometry. For antibody competition assay, different amounts of soluble OKT8 antibody or isotype matched antibody were added to J76CD8SLAM cells before transduction. Two days after transduction, the percentage of GFP^+ cells was determined by flow cytometry and the relative transduction efficiency was normalized to transduction without antibody treatment. To study gene transfer in mixed cultures of target and nontarget cells, J76 and J76CD8 cells were mixed at a ratio of 1:1, and transduced by CD8-LV or VSV-G-LV at an MOI of 2.

To transduce activated PBMCs and purified $CD8^+$ T cells, cells were incubated together with vectors and 4 μg/mL protamine sulfate by centrifugation for 1.5 hours at 800*g*, 32°C, 3 days after cell activation. Then cells were cultured in the presence of 100 IU/mL IL-2. For killing assays and intracellular staining, 12 days after transduction, PBMCs and $CD8^+$ T cells were cultivated for 2 to 3 days in medium containing low amounts of IL-2 (10 IU/mL) to stop proliferation.

Flow cytometry

FACS analysis was performed using the LSRII flow cytometer, and data were analyzed with FACS Diva software (both Becton Dickinson). A phycoerythrin (PE)–conjugated anti-His mAb (Miltenyi Biotec) was used to detect the expression of His-tagged H-αCD8 protein. An allophycocyanin (APC) or PE-conjugated mAb to human CD8 (BD Pharmingen) was used to detect $CD8^+$ cells. Surface expression of TCR-T58 or TCR-D115 in transduced cells was detected by an APC-conjugated anti-mCβ mAb (BD Pharmingen). For functional TCR-T58 or TCR-D115 detection, cells were stained with a PE-labeled HLA-A*0201-Tyr multimer (Beckman Coulter). To identify effector cell activation in the presence of target tumor cells, anti–human CD69-PE mAb (BD Pharmingen) was added to the assay tubes immediately after termination of the killing assay (E:T = 2:1). Isotype control stainings were performed in all experiments.

For intracellular staining to detect GrB and perforin in stimulated effector cells, GrB and perforin specific mAbs were applied according to the manufacturer's instructions (Cytofix/Cytoperm kit with GolgiPlug; BD Bioscience). Stimulations were performed for 4 hours at 37°C in a 96-well plate containing 1×10^5 effector cells and 5×10^4 target cells in the presence of 1 μL/mL BD GolgiPlug. The cells were stained with anti-CD8 and anti-mCβ mAbs, washed, and permeabilized, followed by intracellular staining with anti–human GrB-PE mAb, anti–human perforin-PE mAb, or appropriate isotype controls (BD Pharmingen). Gated $CD8^+$/$TCR\text{-}T58^+$ cells were analyzed for presence of GrB and perforin.

Tumor cell killing assay

Cytotoxic activity of TCR-modified PBMCs or $CD8^+$ T cells was analyzed in FACS-based assays as described.[23] Briefly, 4×10^6/mL target tumor cells were labeled with 1.5 μL/mL calcein violet AM (Invitrogen) for 30 minutes on ice, washed 4 times with RPMI medium, resuspended in T-cell culture medium, and counted. Subsequently, transduced T cells, which were growth arrested by cultivation in 10 IU/mL IL-2–containing medium for 2 to 3 days, were incubated with calcein violet AM-labeled target tumor cells at different E:T ratios. To normalize the number of effectors used and achieve the indicated E:T ratios, the number of $CD8^+$/TCR^+ cells added to targets was determined using the CD8 and HLA-A2–Tyr antibodies previously described. Four hours after coincubation, 0.2 μg propidium iodide (PI; Sigma-Aldrich) were added to stain for dead cells, and the cells were immediately analyzed by flow cytometry. Controls included calcein violet AM-stained target cells as well as PI and

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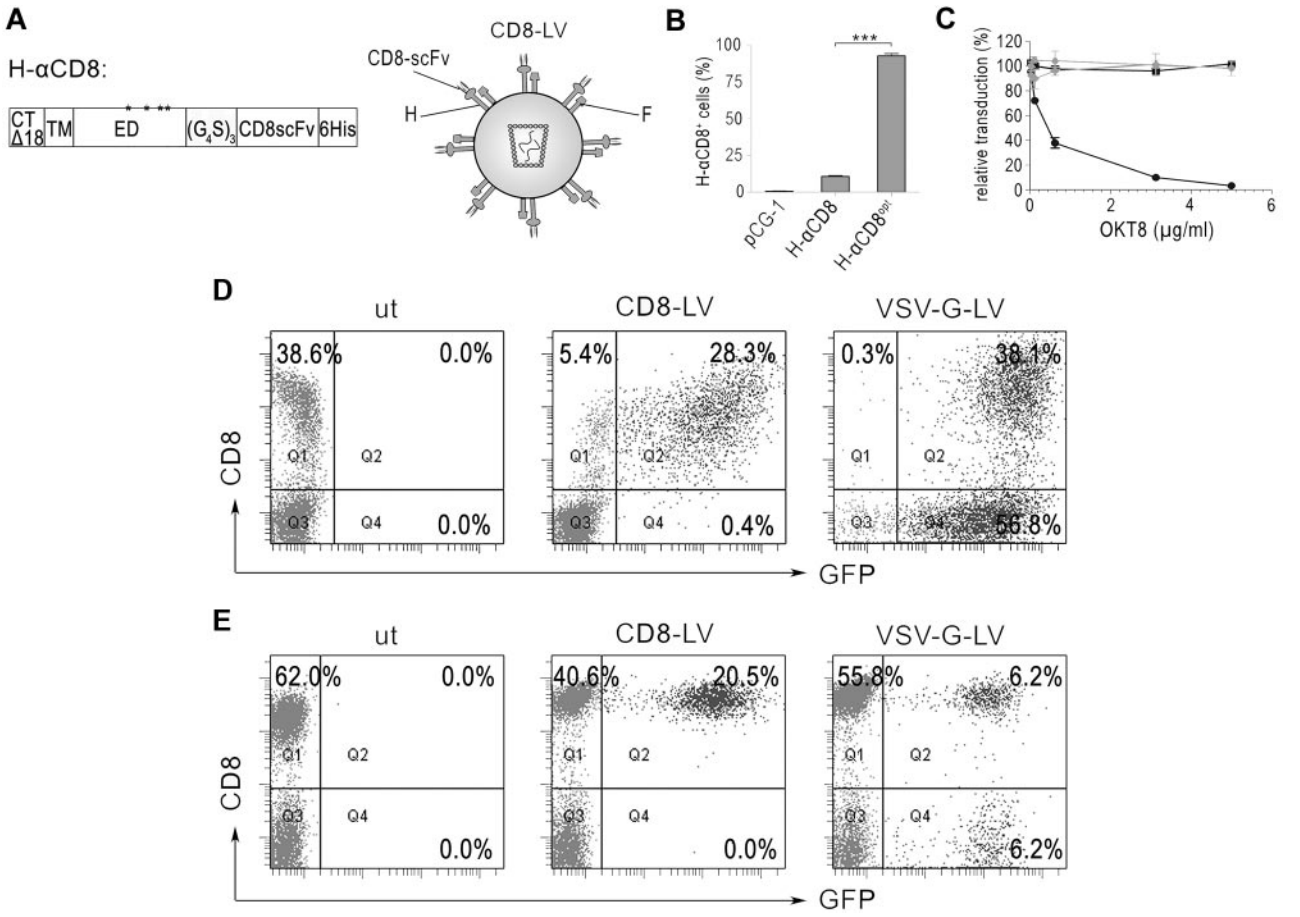

Figure 1. Characterization of CD8-LV. (A) Schematic representation of the H-αCD8 expression construct and CD8-LV. The MV H protein is truncated at its cytoplasmic tail by 18 amino acids (CTΔ18). Four point mutations (Y418A, R533A, S548L, and F549S) in the ectodomain (ED) marked by asterisks abolish natural receptor recognition. The displayed CD8-scFv is linked to H protein via a glycine-serine linker [$(G_4S)_3$]. The transmembrane domain (TM) and a histidine tag (6His) are indicated. (B) Expression plasmids encoding H-αCD8 and H-αCD8^{opt} were transfected into HEK293T cells. Two days later, surface expression of H-αCD8 was quantified by anti-His mAb staining and FACS analysis. The backbone plasmid pCG-1 was used as negative control. Results are expressed as mean ± SEM (n = 3; ***$P < .001$). (C) Transduction of J76CD8SLAM cells by CD8-LV (black circles), VSV-G-LV (gray diamonds), and MV-LV (gray hexagons) was competed on preincubation of cells with the indicated concentration of OKT8 antibody for 20 minutes at 4°C. After 2 days, transduction efficiency was quantified. Isotype control antibody preincubated cells followed by CD8-LV transduction were included as further control (black squares). (D) To study the specificity of CD8-LV, equal numbers of J76 cells and J76CD8 cells were mixed and transduced with the indicated vectors (MOI = 1) or left untransduced (ut). After 48 hours, the percentages of CD8$^+$/GFP$^+$ and CD8$^-$/GFP$^+$ cells were determined by flow cytometry. (E) Freshly isolated human PBMCs were stimulated with 100 IU/mL IL-2 and anti-CD3/CD28 mAbs (1 μg/mL) for 3 days and then transduced with CD8-LV or VSV-G-LV at an MOI of 2. Six days after transduction, PBMCs were analyzed by flow cytometry for GFP expression and CD8 staining.

calcein violet AM–double-stained targets to determine spontaneous lysis. Analysis was performed by gating on the calcein violet AM-labeled target cells, and measuring the PI$^+$ versus PI$^-$ cells. Dead target cells were determined as calcein violet AM and PI double-positive. The percentage of specific tumor cell lysis was calculated by the following equation: (PI$^+$ calcein violet AM$^+$ cells/total number of calcein violet AM$^+$ cells) × 100, corrected for the number of spontaneously lysed targets.

In vivo analysis of CD8 targeting

Experimental mouse work was carried out in compliance with the regulations of the German animal protection law. NOD-*scid-IL2rγ^{null}* mice were bred and maintained under specific pathogen-free conditions and used between 8 and 12 weeks of age. Freshly isolated human PBMCs were stimulated as described for 3 days, and were intraperitoneally injected into NOD-*scid-IL2rγ^{null}* mice. Seven days after PBMC transfer, mice were intraperitoneally injected with CD8-LV-GFP, whereas mice in the control group were intraperitoneally injected with PBS. Mice were killed 7 days after vector particles injection and peritoneal washes were performed using 5 mL PBS. The peritoneal cells were stained by PE-conjugated anti–human CD45 mAb (BD Pharmingen), and the percentage of GFP$^+$ cells within CD45$^+$ human cells was determined by flow cytometry.

Statistical analysis

Results are expressed as mean ± SEM. Data were considered statistically significant for $P < .05$. Differences in H-αCD8 surface expression and in CD69 activation marker expression were evaluated by 1-way ANOVA followed by Tukey test. Percentages of tumor cell lysis mediated by different vector-transduced effector cells in killing assays, and long-term CD8 expression of effector cells were evaluated by 2-way ANOVA to determine significant differences between pairs of groups. Differences in GFP and TCR expression were evaluated by Student *t* test. Differences in GrB and perforin expression were evaluated by paired *t* test. All statistical calculations were done using Prism 5 software (GraphPad).

Results

Generation and characterization of CD8-LV

To generate a CD8-specific scFv, we cloned the coding sequences of the variable domains of the heavy (V_H) and light chain (V_L) from the antibody expressed by the murine hybridoma cell line OKT8.[24] These were assembled to a scFv and then fused to the MV H

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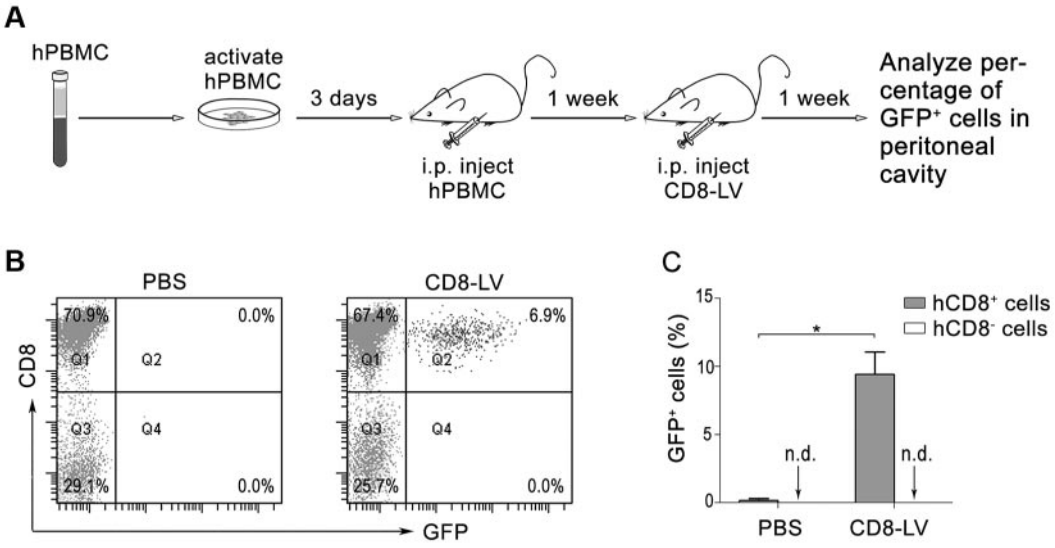

Figure 2. In vivo targeting of CD8⁺ cells. (A) Outline of the experimental setup. Human PBMCs were activated for 3 days after isolation, and 1×10^7 cells were intraperitoneally injected into NOD-*scid-IL2rγ*^{null} mice. One week later, CD8-LV particles (8×10^5 transduction units per mouse), or PBS as a control, were intraperitoneally injected. Peritoneal cells were collected 1 week after vector injection and analyzed by flow cytometry. (B) Representative FACS data demonstrating specific transduction of human CD8⁺ T cells in the peritoneal cavity. Isolated peritoneal cells from PBS or CD8-LV–injected mice were stained with anti–human CD8 mAb, and the percentage of human CD8⁺/GFP⁺ cells was analyzed. (C) Percentages of GFP⁺ cells in hCD8⁺ T cells (gray bars) and hCD8⁻ T cells (white bars) were calculated according to panel B. Results are expressed as mean ± SEM (n = 4; **P* < .05; nd indicates not detectable).

reading frame resulting in plasmid pCG-H-αCD8 encoding CD8-scFv fused H protein (H-αCD8; Figure 1A; supplemental Figure 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). When pCG-H-αCD8 was expressed in HEK293T cells we observed only moderate cell surface expression levels of H-αCD8 protein, which were insufficient to release high-titer vector particles (Figure 1B). In an attempt to enhance vector titers, we identified the 6 complementarity determining regions (CDRs) and grafted them into the framework of the most similar human variable domain sequences (supplemental Figure 1). This humanized CD8-specific scFv, CD8-scFv^{opt}, specifically bound CD8⁺ but not CD8⁻ cells (supplemental Figure 2). The corresponding H-αCD8^{opt} protein was efficiently expressed on the cell surface (Figure 1B) and allowed the generation of high-titer CD8-LV stocks.

To assess whether gene transfer mediated by CD8-LV was because of the displayed CD8-specific scFv, we transduced CD8⁺ cells in presence of OKT8. As shown in Figure 1C, OKT8 dramatically reduced CD8-LV–mediated transduction in a concentration-dependent manner, whereas the addition of isotype control antibody had no effect. Moreover, the presence of OKT8 did not influence transduction mediated by MV-LV,[25] carrying nonblinded MV glycoproteins, or VSV-G-LV. This result demonstrates that transduction mediated by CD8-LV was because of the displayed scFv. Next, we evaluated the specificity of CD8-LV in mixed cultures of human CD8⁺ and CD8⁻ T-cell lines (Figure 1D) or on human PBMCs (Figure 1E). Although VSV-G-LV transduced both CD8⁺ T cells and CD8⁻ T cells with an overall similar efficiency, CD8-LV only transduced the CD8⁺ cells but left CD8⁻ T cells untransduced. Of note, MV-LV transduced both CD8⁺ and CD4⁺ cells in freshly isolated PBMCs with a preference for CD4⁺ cells (Frecha et al[26] and data not shown), which further proved that the specificity of CD8-LV was caused by the displayed scFv and not simply by the MV envelope. To assess the stability of gene transfer and to exclude any toxic effects exerted by CD8-LV, transduced cells were cultivated and the percentages of GFP⁺ cells were followed more than time. The data demonstrate that the level of GFP⁺ cells was stable during the observation period in cell lines as well as PBMCs (supplemental Figure 3).

CD8⁺ T cell specific gene transfer in vivo

The in vivo targeting potential of CD8-LV was studied in immunodeficient mice grafted with human PBMCs. Stimulated human PBMCs were injected intraperitoneally into NOD-*scid-IL2rγ*^{null} mice (Figure 2A). CD8-LV or PBS as control was intraperitoneally injected 1 week after PBMC transplantation. After another week, approximately two-thirds of the cells were CD8⁺ in both PBS and CD8-LV injected mice (Figure 2B). On average, approximately 10% of the cells were GFP⁺ although only a single dose of vector particles had been applied. Remarkably, all GFP⁺ cells also stained positive for human CD8 in each of the mice investigated (Figure 2C). Thus, CD8-LV targeted human CD8⁺ cells in a humanized mouse model specifically and efficiently.

Efficient delivery of therapeutic transgenes to CD8⁺ cells

Next, we investigated whether tumor-reactive TCRs can be efficiently delivered into CD8⁺ T cells using CD8-LV. Because VSV-G-LV is the most frequently used LV vector type, we compared CD8-LV to this sort of gold standard vector. In all upcoming experiments, the 2 vector types just differ in their envelope glycoproteins, whereas vector core and transfer vector are identical. Two tyrosinase (Tyr) peptide-specific, HLA-A2–restricted TCRs, TCR-T58 and TCR-D115, were selected, which differ 100-fold in their affinity.[18] After transferring the TCR genes into human PBMCs by CD8-LV or VSV-G-LV, TCR expression was quantified by HLA-A2–Tyr multimer staining. CD8-LV–transduced PBMCs expressed the TCR exclusively in the CD8⁺ cell fraction, whereas on VSV-G-LV transduction, both, CD8⁺ and CD8⁻ cells were TCR⁺ (Figure 3A-B). Of note, the majority (more than 90%) of CD8⁻ cells stained positive for CD4 (data not shown), meaning VSV-G-LV transduced both CD8⁺ and CD4⁺ T cells, whereas CD8-LV left CD4⁺ cells untransduced. To compare the transduction efficiency of CD8-LV and VSV-G-LV on

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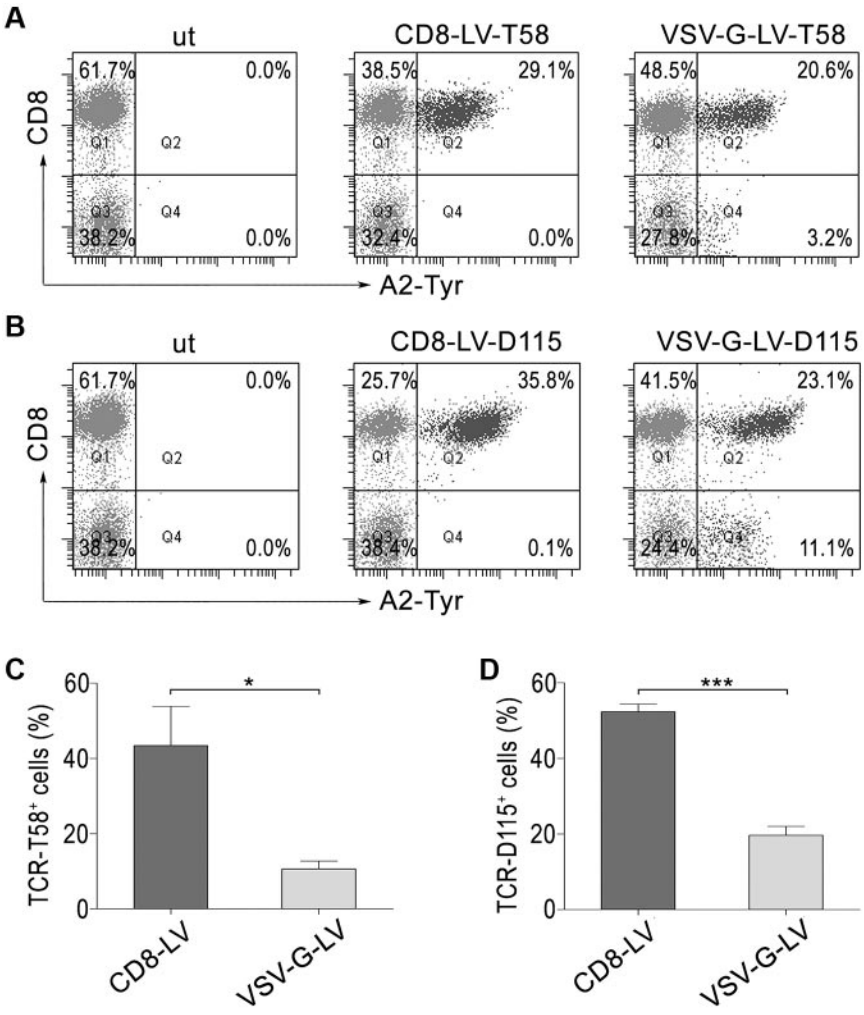

Figure 3. Efficient TCR gene delivery by CD8-LV. Activated human PBMCs were left untransduced (ut) or transduced by CD8-LV or VSV-G-LV harboring either the TCR-T58 (A), or the TCR-D115 transgene (B) at an MOI of 2. Transduced PBMCs were analyzed after 7 days for TCR expression using HLA-A2-Tyr multimer staining. Percentages of $CD8^+$/multimer$^+$ cells are displayed in the upper right gate. (C-D) Purified human $CD8^+$ T cells were activated for 3 days and transduced by CD8-LV or VSV-G-LV at an MOI of 2. Seven days later, percentages of TCR-$T58^+$ (C) or TCR-$D115^+$ cells (D) were determined by flow cytometry. Results are expressed as mean ± SEM (n = 3; $^*P < .05$; $^{***}P < .001$).

monocultures of $CD8^+$ cells, we purified $CD8^+$ T cells by negative selection and transduced the cells with both vector types at the same MOI as determined on Molt cells. As shown in Figure 3C-D, both TCR-T58 and TCR-D115 expression was superior using CD8-LV compared with VSV-G-LV–mediated gene transfer. These data demonstrate that CD8-LV specifically delivered more complex genes, such as TCR α and β-chains into $CD8^+$ T cells and was more efficient than VSV-G-LV.

Efficient tumor cell killing by CD8-LV TCR gene-modified T cells

Next, we investigated the cytotoxic potential of CD8-LV and VSV-G-LV–transduced T cells expressing tumor cell-reactive TCRs. To adjust for the more efficient TCR gene delivery mediated by CD8-LV, we added relatively more VSV-G-LV–transduced cells to reach equivalent effector to target (E:T) ratios in the tumor cell killing assay for both types of vector-transduced cells. Both, T58 and D115 TCR-transduced cells specifically recognized and killed Tyr^+/$HLA\text{-}A2^+$ tumor cells, but not Tyr^- or $HLA\text{-}A2^-$ control tumor cells. Remarkably, CD8-LV–transduced cells reproducibly killed tumor cells more efficiently than VSV-G-LV–transduced cells (Figure 4A, supplemental Figure 4). To achieve a similar level of tumor cell killing as that mediated by CD8-LV TCR-transduced T cells, between 5 and 10 times more VSV-G-LV TCR-transduced effector cells had to be applied (Figure 4A; compare 1:1 and 5:1 ratios of the CD8-LV-T58 samples with 5:1 and 20:1 of VSV-G-LV-T58 samples, respectively).

Although in the assay described in the previous paragraph the numbers of TCR^+ T cells were equal for both vector types, significantly more noneffector cells were present in the cell mixtures for the VSV-G-LV than for the CD8-LV samples. Thus, redundant noneffectors might have potentially obstructed the effector T cells to recognize and kill tumor cells during the 4-hour incubation period of the killing assay. To exclude that the observed enhanced killing mediated by CD8-LV–transduced cells was simply because of the experimental setup, we transduced PBMCs with VSV-G-LV at an MOI of 100, thereby reaching 35.8% of $CD8^+$/TCR-$T58^+$ cells, a percentage almost identical to that obtained with CD8-LV at an MOI of 2 (36.3%). In addition, PBMCs were transduced with VSV-G-LV at an MOI of 2 (16.8% of $CD8^+$/TCR-$T58^+$ cells), and the number of effector cells and E:T ratios were normalized as before. As shown in Figure 4B, there was no difference in the killing efficiency between VSV-G-LV TCR-transduced cells at MOI of 2 and 100. Thus, redundant noneffector cells did not influence the killing efficiency in our experimental setup. CD8-LV TCR-transduced cells again displayed a substantially enhanced killing activity, which must be because of specific properties of the CD8-LV vector rather than its enhanced $CD8^+$ cell transduction efficiency.

It is widely accepted that $CD4^+$ helper T cells augment $CD8^+$ T-cell–mediated tumor control and eradication, although in a short-term killing assay this helper function may be limited.[27-29] To exclude any effect mediated by $CD4^+$ T cells, we purified $CD8^+$ T cells from PBMCs, transduced them with both vector types and performed the killing assay. Similarly as with PBMCs, TCR-T58–modified $CD8^+$ T cells killed tumor cells more efficiently when the

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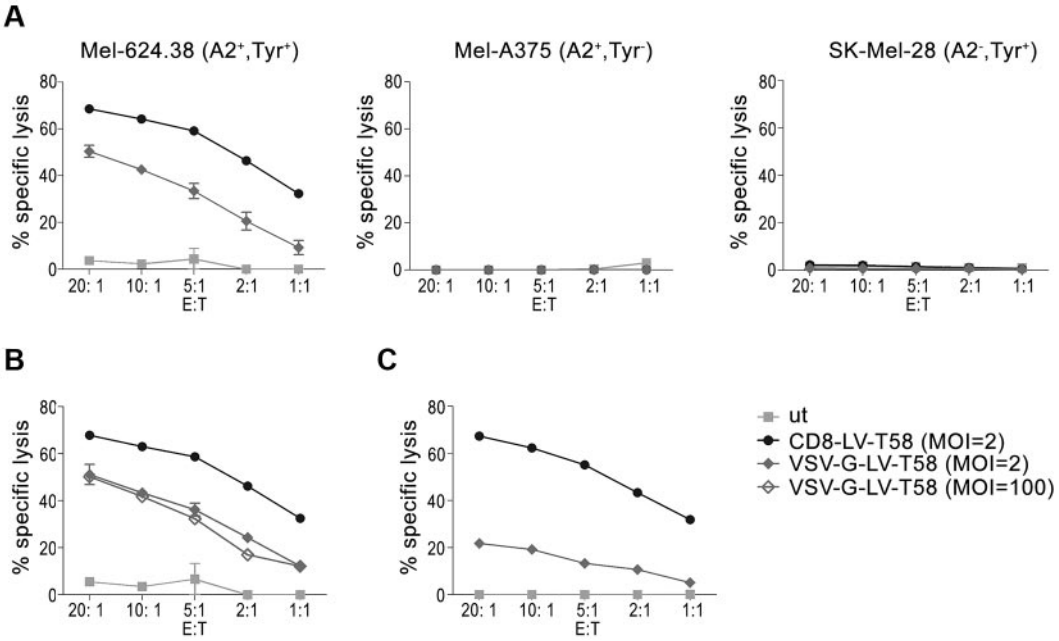

Figure 4. Efficient tumor cell lysis by CD8-LV TCR-transduced T cells. Activated PBMCs (A-B) or purified CD8$^+$ T cells (C) were transduced with CD8-LV or VSV-G-LV at the indicated MOIs, and expanded in 100 IU/mL IL-2–containing medium. On day 12, transduced cells were transferred into 10 IU/mL IL-2–containing medium for 2 to 3 days, and TCR-T58 expression was determined by HLA-A2-Tyr multimer staining (data not shown). Identical numbers of CD8$^+$/TCR-T58$^+$ cells were applied as effector cells and used to normalize E:T ratios. (A) TCR-T58-modified PBMCs were incubated with Mel-624.38 cells or as controls with Mel-A375 or SK-Mel-28 cells at the indicated E:T ratios for 4 hours, and target cell killing was quantified using a FACS-based cytotoxicity assay. Mean values ± SEM calculated from 3 independent experiments are shown. The difference between CD8-LV-TCR-T58 and VSV-G-LV-TCR-T58 (MOI = 2) treated groups is highly significant ($P < .0001$). (B) TCR-T58–expressing PBMCs transduced by the indicated vectors at the indicated MOIs were incubated with Mel-624.38 cells for 4 hours. Mean values ± SEM calculated from 2 independent experiments are shown. The difference between CD8-LV-TCR-T58 and VSV-G-LV-TCR-T58 (MOI = 100) treated groups is significant ($P < .0001$), whereas the difference between VSV-G-LV-TCR-T58 (MOI = 2) and VSV-G-LV-TCR-T58 (MOI = 100) treated group is not ($P > .05$). (C) Purified CD8$^+$ T cells transduced by the indicated vectors were incubated with Mel-624.38 cells for 4 hours, and specific target-cell lysis was determined. Representative results from 1 of 2 independent experiments with T cells from 2 different donors are shown. The difference between CD8-LV-TCR-T58 and VSV-G-LV-TCR-T58 (MOI = 2) treated groups is significant ($P < .001$).

cells had been transduced with CD8-LV (Figure 4C). This result was confirmed with the low affinity tyrosinase-reactive TCR-D115. Also in this case, CD8-LV–transduced CD8$^+$ T cells were superior in cell killing compared with VSV-G-LV–transduced T cells (supplemental Figure 4). Furthermore, the enhanced killing activity of CD8-LV TCR-transduced T cells correlated positively with the levels of granzyme B (GrB) and perforin expression. As summarized in Figure 5, VSV-G-LV–transduced cells expressed lower amounts of GrB and perforin than CD8-LV–transduced cells.

Increased CD8 coreceptor expression levels in CD8-LV–transduced cells

To further investigate the molecular mechanism causing the enhanced antitumoral response of CD8-LV–transduced cells, transgenic TCR and surface CD8 expression levels were determined before performing the killing assay. Because the same number of effector cells transduced by CD8-LV and VSV-G-LV was used, differences in the amounts of transgenic TCR per cell might have affected effector cell activation and target cell lysis. Therefore, we quantified the TCR-T58 surface density on effector cells. As shown in Figure 6A, no difference was observed in surface TCR-T58 levels between CD8-LV and VSV-G-LV–transduced effector cells. Thus, differences in transgenic TCR densities can be ruled out as causative for the enhanced cytotoxic activity mediated by CD8-LV.

Previous studies have shown that CD8 is important for CD8$^+$ T-cell signaling,[30] and changes in CD8 levels can affect T-cell function.[31] Thus, we next quantified the CD8 surface density in

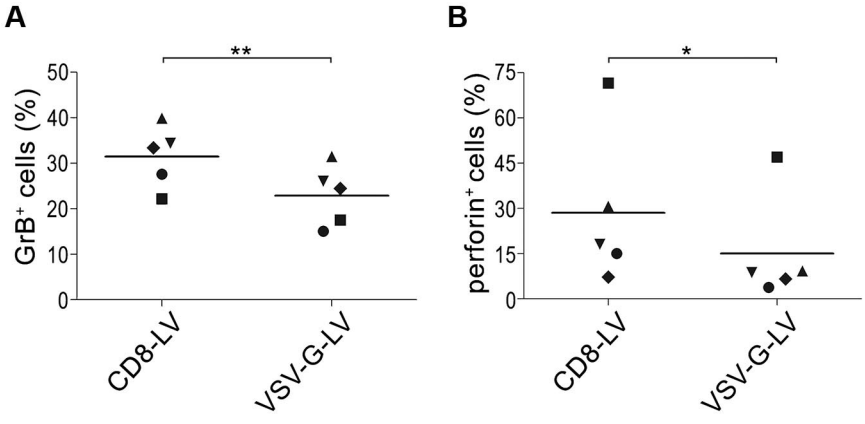

Figure 5. Granzyme B and perforin in CD8-LV TCR-transduced T cells. PBMCs were treated as described in Figure 4. CD8-LV-TCR-T58 or VSV-G-LV-TCR-T58–transduced PBMCs (MOI = 2) were incubated with Mel-624.38 (E:T = 2:1) in the presence of GolgiPlug. After 4 hours, cells were surface stained with anti-CD8 and anti-mCβ mAbs to determine the total number of effector cells (CD8$^+$/TCR-T58$^+$), and intracellularly stained with specific mAbs to determine the percentage of GrB (A) or perforin (B) expressing cells. Data are from 5 different donors. Individual symbols represent individual donors to visualize trends per donor. Bars represent median values (*$P < .05$; **$P < .01$).

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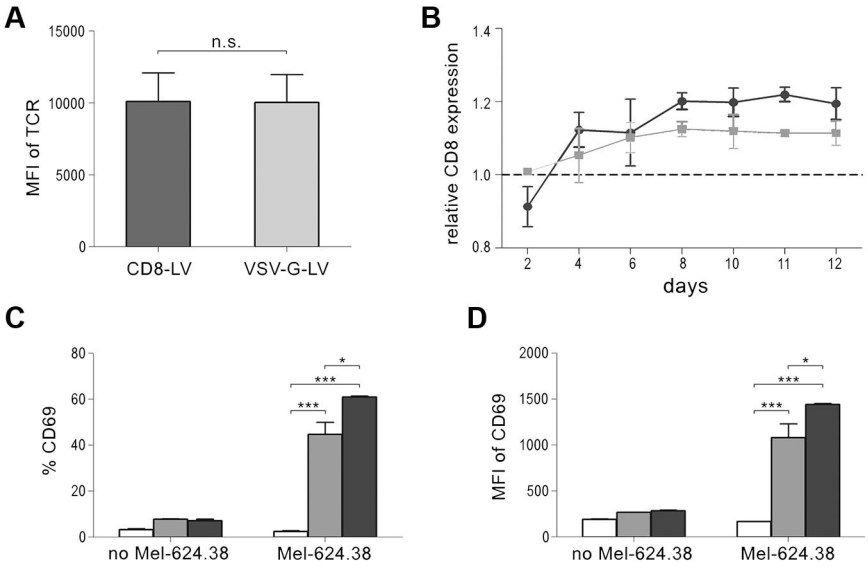

Figure 6. Increased CD8 density and enhanced activation of CD8-LV TCR-transduced cells. (A) Activated PBMCs were transduced with CD8-LV-TCR-T58 or VSV-G-LV-TCR-T58 at an MOI of 2. Approximately 2 weeks later, TCR expression levels were determined using HLA-A2-Tyr multimers in the presence of anti-CD8 mAb. Mean fluorescent intensities (MFI) of the TCR specific signal are shown for the transduced effector cells of each vector type. Results are expressed as mean ± SEM (n = 10). ns indicates not significant. (B) Activated PBMCs were transduced with CD8-LV-TCR-T58 (dark gray line) or VSV-G-LV-TCR-T58 (light gray line) at an MOI of 2. Then, $CD8^+$/TCR-$T58^{high}$ cells were gated (supplemental Figure 5), and CD8 surface densities were determined at the indicated days as relative MFI normalized to that of untransduced cells. Results are expressed as mean ± SEM (n = 3). The difference between CD8-LV-TCR-T58 and VSV-G-LV-TCR-T58 treated groups is significant ($P < .05$). (C-D) Activated PBMCs were left untransduced (white bars), transduced by VSV-G-LV-TCR-T58 (gray bars), or CD8-LV-TCR-T58 (black bars). Cells were then cultivated for 12 days, before they were transferred into IL-2 (10 IU/mL) containing medium for 3 days. Effector cells were incubated with or without Mel-624.38 cells for 4 hours and stained with anti-CD8, anti-mCβ, and anti-CD69 mAbs or appropriate isotype controls. The gate determining $CD69^+$ cells was set according to the isotype control antibody stained untransduced cells. Overall percentage of $CD69^+$ cells (C), and MFI of $CD69^+$ cells (D) among $CD8^+$/TCR-$T58^{high}$ cells are shown. Results are expressed as mean ± SEM (n = 3; $^*P < .05$; $^{***}P < .001$).

$CD8^+$/TCR-$T58^{high}$ effector cells (supplemental Figure 5), where a sufficient number of transgenic TCRs were expressed to exceed the activation threshold required for cytokine release and cytotoxic activity. As shown in Figure 6B, the CD8 density was slightly but significantly enhanced in cells transduced by CD8-LV compared with VSV-G-LV–transduced cells. This observation was consistently made at several time points over a 12-day cultivation period with the only exception shortly after transduction (Figure 6B).

Furthermore, we quantified expression of the T-cell early activation marker CD69 after cocultivating effector and target cells. In absence of target cells, untransduced T cells as well as CD8-LV and VSV-G-LV–transduced T cells expressed only low levels of CD69 (Figure 6C-D). After adding target cells, both types of vector transduced cells displayed significantly higher levels of CD69 than untransduced T cells. Importantly, CD8-LV–transduced cells thereby expressed higher amounts of CD69 than VSV-G-LV–transduced cells. There were significantly more $CD69^+$ cells, and also the CD69 density was increased (Figure 6C-D). These data suggest that the enhanced antitumoral activity of CD8-LV–transduced cells was because of an enhanced activation and increased CD8 expression levels.

Discussion

We present here the first gene vector (CD8-LV) that delivers genes precisely and exclusively to $CD8^+$ T cells. The vector is derived from lentiviral vectors, which exhibit several unique advantages, such as the ability to transduce minimally stimulated[32,33] or nonstimulated T cells,[22,26] as well as a preference for safer integration sites compared with γ-retroviral vectors.[34] LVs have therefore recently been suggested as attractive alternative to γ-retroviral vectors, which are at present commonly used for TCR gene transfer.[1-3] The selectivity of CD8-LV for $CD8^+$ cells relies on the high specificity of antibody-antigen recognition. As targeting domain, a scFv antibody fragment was generated from the broadly used mAb OKT8, which is specific for the α-chain of CD8. Notably, the scFv as such was not suited as targeting domain. Only by grafting its CDR regions into a human scFv framework we were able to convert the OKT8-derived scFv into a fully functional targeting domain. It is conceivable that this engineering step improved folding and enhanced stability of the scFv thereby increasing expression levels and cell-surface transport of the H-scFv fusion protein.[11]

CD8-LV discriminated between $CD8^+$ and $CD8^-$ cells with virtually absolute specificity, although retaining high transduction efficiency and stable gene delivery. Remarkably, this was not only the case ex vivo on primary cells, but also in vivo where CD8-LV selectively transferred genes into $CD8^+$ T cells in NOD-*scid*-*IL2rγ*^{null} mice transplanted with human PBMCs. This suggests that CD8-LV will be applicable for in vivo gene transfer strategies. CD8-LV maintained its specificity and efficiency even when delivering more complex genes, such as TCR encoding sequences. Thus, CD8-LV is the first transfer vector that delivers genes exclusively to $CD8^+$ cells. In principle, target cells of CD8-LV include all $CD8\alpha^+$ cells, which are besides $CD8^+$ T cells dendritic cells and natural killer cells.[35] Although these cells may form an attractive target for antigen delivery, they will neither survive the ex vivo expansion nor express introduced TCR genes and therefore are not relevant for TCR gene therapy.

Although titers of CD8-LV stocks were on average approximately 100-fold lower than those of VSV-G-LV, CD8-LV was more efficient than VSV-G-LV in delivering TCR genes to $CD8^+$ T cells. An unexpected and remarkable outcome of this study was the substantially enhanced tumor-cell killing activity of CD8-LV versus VSV-G-LV TCR–transduced effector T cells. To achieve a

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similar efficiency in target cell lysis, approximately 5 to 10 times more effector cells transduced with VSV-G-LV were required. This difference became even more pronounced when a pure culture of $CD8^+$ T cells was transduced, and, although to a lower extend, was also observed when the low affinity TCR-D115 was applied. Straightforward explanations such as differences in the amounts of introduced TCR expressed on the cell surface or the sensitivity of modified T cells toward activation-induced cell death on tumor antigen restimulation (supplemental Figure 6) were excluded as being responsible for the different tumor killing activity of CD8-LV versus VSV-G-LV–transduced cells. These data demonstrate that this effect was not limited to a particular type of TCR, and was not because of the $CD8^+$ cell targeting capability of CD8-LV. Rather, it must have been a consequence of CD8-LV particle binding to CD8 followed by membrane fusion and cell entry.

Human CD8 is a heterodimeric protein that binds to MHC class I molecules.[36] Although its binding affinity to pMHC is weaker than that of TCRs, it is well established that the interaction between CD8 and MHCI is critical for T-cell activation and lysis of target cells.[37-40] Several previous studies have confirmed that higher CD8 levels (1) positively correlate with the sensitivity of TCRs for antigens and the cytolytic activity of T cells,[41,42] (2) enhance the avidity of TCRs for the p-MHCI complex,[43] and (3) enhance the expression of perforin and granzymes resulting in increased cytotoxicity.[44] We observed in CD8-LV but not in VSV-G-LV TCR-transduced T cells a small subpopulation of cells exhibiting a slight but significant up-regulation of CD8 surface expression. One likely explanation is that CD8-LV preferentially transduced T cells with the highest CD8 density. We previously found that gene transfer efficiency positively correlated with target receptor density for CD105-LV[10] and Her2neu-LV.[11] Alternatively, binding of CD8-LV to CD8, similarly as antibody-antigen binding, may have led initially to CD8 down-regulation and then to an over-compensated up-regulation of CD8 expression. In fact, CD8 levels transiently declined 2 days after CD8-LV transduction, but then steadily increased.

A recent study investigating systematically the influence of a panel of CD8-specific mAbs on $CD8^+$ T cell effector functions revealed a unique property for OKT8: of 8 antibodies tested only OKT8 induced effector functions of $CD8^+$ cells.[30] Although a monovalent Fab fragment of OKT8 failed to activate $CD8^+$ T cells, the corresponding bivalent $F(ab')_2$ fragment retained some ability to enhance pMHCI tetramer staining.[30] This suggests that for activation the Fc part of the antibody itself is not essential, whereas multiple CD8 contacts are indeed important. Lentiviral vectors produced in packaging cells can contain approximately 100 envelope proteins per particle.[45] We found around 80 MV H proteins per particle by quantitative mass spectrometry (N. Kirsch and C.J.B., unpublished data, December 20, 2010). It is thus probable that multiple scFv/CD8 contacts are formed during entry of a single CD8-LV particle into a single $CD8^+$ effector cell.

Moreover, we found increased GrB and perforin levels in CD8-LV TCR-transduced T cells compared with VSV-G-LV–transduced cells, although both cell types showed equivalent IFN-γ and TNF-α levels (supplemental Figure 7). Although these results contrast with previous reports indicating a direct correlation between IFN-γ release and cytotoxic activity of CTL clones and TCR gene-modified T cells,[46-48] they are in line with recent findings that acute killing is characterized by GrB/perforin-induced cytotoxicity, and that cytotoxicity may not necessarily correlate with cytokine release, especially IFN-γ production.[49] Also OKT8-induced effector functions occur in absence of cytokine release.[30]

Taken together, it is probable that at least 2 mechanisms contribute to the enhanced tumor cell killing induced by CD8-LV: (1) activation of effector functions by binding of the displayed OKT8-derived scFv molecules to CD8 on the surface of $CD8^+$ T cells via multiple contacts; and (2) up-regulation of CD8 surface expression in a small subpopulation of TCR^+ cells. Accordingly, CD8-LV–transduced T cells would better stabilize the TCR/p-MHC complex and reduce the TCR-dependent activation threshold, leading to a stronger T-cell activation, more GrB and perforin production, and more efficient tumor cell lysis compared with T cells transduced by the conventional lentiviral vector. To further test this hypothesis, the stabilization of the TCR/p-MHC complex between CD8-LV and VSG-LV–transduced cells can be directly compared by tetramer decay assay.

In summary, our study describes the first $CD8^+$ cell targeted gene vector and demonstrates its successful application for in vivo targeting and TCR gene therapy. The finding, that CD8 targeting enhances the antitumoral activity of TCR-modified T cells, underscores the importance of the CD8 coreceptor function in T-cell activation. A positive safety profile provided, this finding also suggests that CD8-LV has the potential to improve the efficacy of TCR gene therapy for cancer. Beyond that, exclusive gene transfer to $CD8^+$ cells opens up novel options for therapeutic strategies of other diseases such as chronic virus infections for which natural TCRs or CARs can be used. Moreover, by local injection of CD8-LV at sites of inflammation it may be possible to deliver, for example suicide genes to selectively eliminate $CD8^+$ cells.

Acknowledgments

The authors thank Jörg Kirberg (Paul-Ehrlich-Institut, Langen, Germany) for helpful discussions and Els Verhoeyen (Université de Lyon and Ecole Normale Supérieure de Lyon, Lyon, France) for critical reading of the paper.

This work was supported by a grant of the Priority Program "Mechanisms of gene vector entry and persistence" of the Deutsche Forschungsgemeinschaft (BU-1301/2-2, UC 4/5-2) and by the LOEWE Center for Cell and Gene Therapy Frankfurt funded by Hessisches Ministerium für Wissenschaft und Kunst (III L 4-518/17.004 [2010] to C.J.B.). Q.Z. is supported by the China Scholarship Council and is an associated member of DFG Graduiertenkolleg GRK1172. I.E. and W.U. are supported by the German Research Council (SFB TR36).

Authorship

Contribution: Q.Z. designed and performed experiments and contributed to writing the paper; I.C.S. and A.H. cloned and designed CD8-scFv; I.C.S. and S.K. performed experiments, contributed to supervision of work, and wrote the paper; P.B. and I.E. performed experiments; K.S., W.S.W., and A.S. contributed vital reagents and protocols; W.U. contributed to the project design and wrote the paper; and C.J.B. conceived and designed the study, acquired grants, supervised work, and wrote the paper.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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